AD_____

Award Number: W81XWH-11-1-0458

TITLE: Toward Development of a Food-Based Genetic Approach to Overcoming Food Allergies

PRINCIPAL INVESTIGATOR: Jixun Zhan

CONTRACTING ORGANIZATION: Utah State University
Logan, UT 84322-1415

REPORT DATE: October 2012

TYPE OF REPORT: Revised Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2012	Revised Final	30 September 2011- 29 September 2012

4. TITLE AND SUBTITLE
Toward Development of a Food-Based Genetic Approach to Overcoming Food Allergies

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0458

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Jixun Zhan

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

E-Mail: jixun.zhan@usu.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Utah State University
Logan, UT 84322-1415

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

.
Curcuminoids are plant natural products that have shown promising anti-allergic activities. They have been used in Asian traditional medicine and food spice for centuries and have been confirmed to be safe. In this study, we have reconstituted curcuminoid biosynthesis in Escherichia coli by heterologous expression of the biosynthetic enzymes. We have also established an effective expression platform for functional reconstitution of natural product biosynthetic enzymes in lactic acid bacteria (LAB) using green fluorescent protein as a reporter. A colony PCR technique has been developed to efficiently screen correct LAB transformants. A series of expression plasmids thatharbor curcuminoid biosynthetic genes and other natural product biosynthetic enzymes has been constructed for expression in LAB.

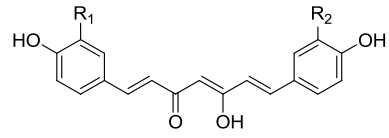
15. SUBJECT TERMS- Anti-allergic, Natural products, Engineered biosynthesis, Lactic acid bacteria

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	13	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction

Nature provides a variety of small molecules that have found important medical uses. These compounds are secondary metabolites synthesized by plants, animals and microorganisms. Some bioactive natural products have been developed into important drugs that are widely used, such as paclitaxel (anticancer), tetracycline (antibacterial) and lovastatin (anti-cholesterol). Many other molecules have also been confirmed to be efficacious and safe, but their use has been limited by their water solubility and bioavailability. This is exemplified by curcuminoids (Fig. 1), a group of beneficial plant natural products from turmeric. These natural products have long been used as a food spice (curry) and complementary and alternative medicine (CAM) in Asia.[1-4] They possess various bioactivities and can be used for the treatment of different diseases such as allergy, asthma, cancer and Alzheimer's disease, while providing prevention against oxidative damage in normal cells.[5-10] However, the use of curcuminoids has been hindered by their poor water solubility and low bioavailability. A large quantity of curcuminoids has to be taken for therapeutic applications. For example, a recent clinical trial on the effect of supplemental oral curcumin in patients with atopic asthma requires patients to take 2,000 mg of curcumin every day.[11]



Curcumin $R_1 = R_2 = OCH_3$
Demethoxycurcumin $R_1 = OCH_3$, $R_2 = H$
Bisdemethoxycurcumin $R_1 = R_2 = H$

Fig. 1 Three natural curcuminoids.

Lactic acid bacteria (LAB) are one of the most common types of probiotics. They are widely used in the food industry to make fermented products. Most LAB species are generally regarded as safe (GRAS) organisms by the U.S. Food and Drug Administration. The probiotic properties and ability to survive passage through the human gastrointestinal tract make LAB a potential platform for developing new therapies. Studies have shown that LAB from yogurt can become normal components of intestinal microflora; they benefit human health by providing protection from invasion of pathogenic bacteria and producing bioactive metabolites.[12-15] It is reasonable to hypothesize that engineered LAB can be delivered to and inhabit the human body to become efficient cell factories that will continuously provide antiallergic molecules *in situ* for preventive treatment of food allergies.

This project aims to engineer the biosynthesis of curcuminoid in a strain of LAB. Two specific objectives were originally proposed: (1) engineer biosynthesis of curcuminoids in *Streptococcus salivarius subsp. thermophilus*; and (2) comparative analysis of yogurt products resulting from the wide type and engineered strain. In this funding period, we have found that *Lactobacillus casei* is more friendly to genetic engineering than *S. salivarius subsp. thermophilus*. Thus, we decided to choose *L. casei* as the LAB host for curcuminoid biosynthesis. Because natural product biosynthesis has never been done in LAB, we have used the one-year funding period to establish a biosynthetic platform for curcuminoid production in *L. case* and made significant progress in the proposed research. Briefly, we have reconstituted the biosynthesis of curcuminoids in *Escherichia coli*, developed a novel visible reporter assay for phenylalanine ammonia-lyase (PAL), tested a variety of *E. coli*/LAB shuttle vectors, expressed green fluorescent protein (GFP) in LAB using pMG36e, developed a colony polymerase chain reaction (PCR) technique for analysis of correct LAB transformants, and constructed a series of expression plasmids for the biosynthesis of different natural products including curcuminoids.

Body

Task 1: Engineer biosynthesis of curcuminoids in *S. salivarius subsp. thermophilus*

1. Reconstitution of curcuminoid biosynthesis in *E. coli*

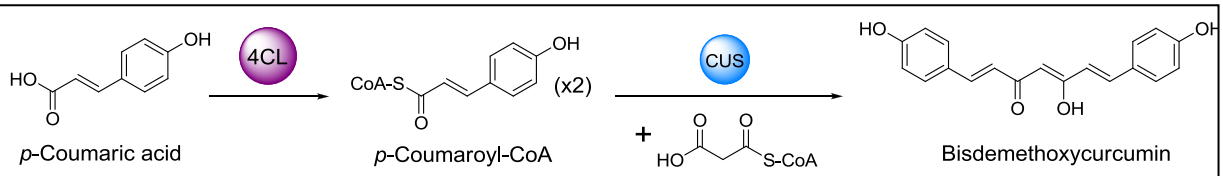


Fig. 2 Biosynthesis of bisdemethoxycurcumin.

Curcuminoids are synthesized by a type III polyketide synthase (PKS) from *Oryza sativa*, CUS (curcuminoid synthase), from *p*-coumaroyl-CoA and malonyl-CoA. An example of curcuminoid biosynthesis is shown in Fig. 2. First, a 4-coumarate:CoA ligase (4CL) synthesizes an aromatic CoA ester such as *p*-coumaroyl-CoA from CoA and the corresponding acid. CUS then synthesizes a diketide-CoA from a molecule of malonyl-CoA and a unit of aromatic CoA ester. It then utilizes the nucleophilic water to terminate the initial polyketide chain elongation at the diketide stage.[16] Cleavage of CoA from the diketide-CoA yields the corresponding β-keto acid that is used by CUS as the second extender unit to afford bisdemethoxycurcumin.[17] In order to engineer the biosynthesis of curcuminoids in LAB, it is necessary to get functional biosynthetic enzymes. Thus, we first reconstituted the biosynthesis of curcuminoids in *E. coli*. The CUS gene was cloned from the cDNA of *O. sativa*, and a gene encoding 4CL (named 4CL1) was amplified from *Arabidopsis thaliana* (Fig. 2). These two genes were ligated into pET28a and pACYCDuet-1, yielding pSW24 and pSW42 (Table 1), respectively. Co-transformation of these two plasmids into *E. coli* BL21(DE3) led to the engineered strain *E. coli* BL21(DE3)/pSW24+pSW42. The strain was cultured in LB supplemented with kanamycin and chloramphenicol. When the OD_{600} reached ~0.45, 200 mM IPTG was added to induce the expression of the proteins. In the meantime, 40 g/l glucose, 25 g/l $CaCO_3$ and 3 mM *p*-coumaric acid were added into the fermentation broth. The culture was maintained at 25 °C for 48 hours and then extracted with equal volume of ethyl acetate. After evaporation of the solvent, a yellow solid was observed (Fig. 3A). HPLC analysis at 420 nm indicated a major peak at 17.3 min (Fig. 3B). The UV spectrum

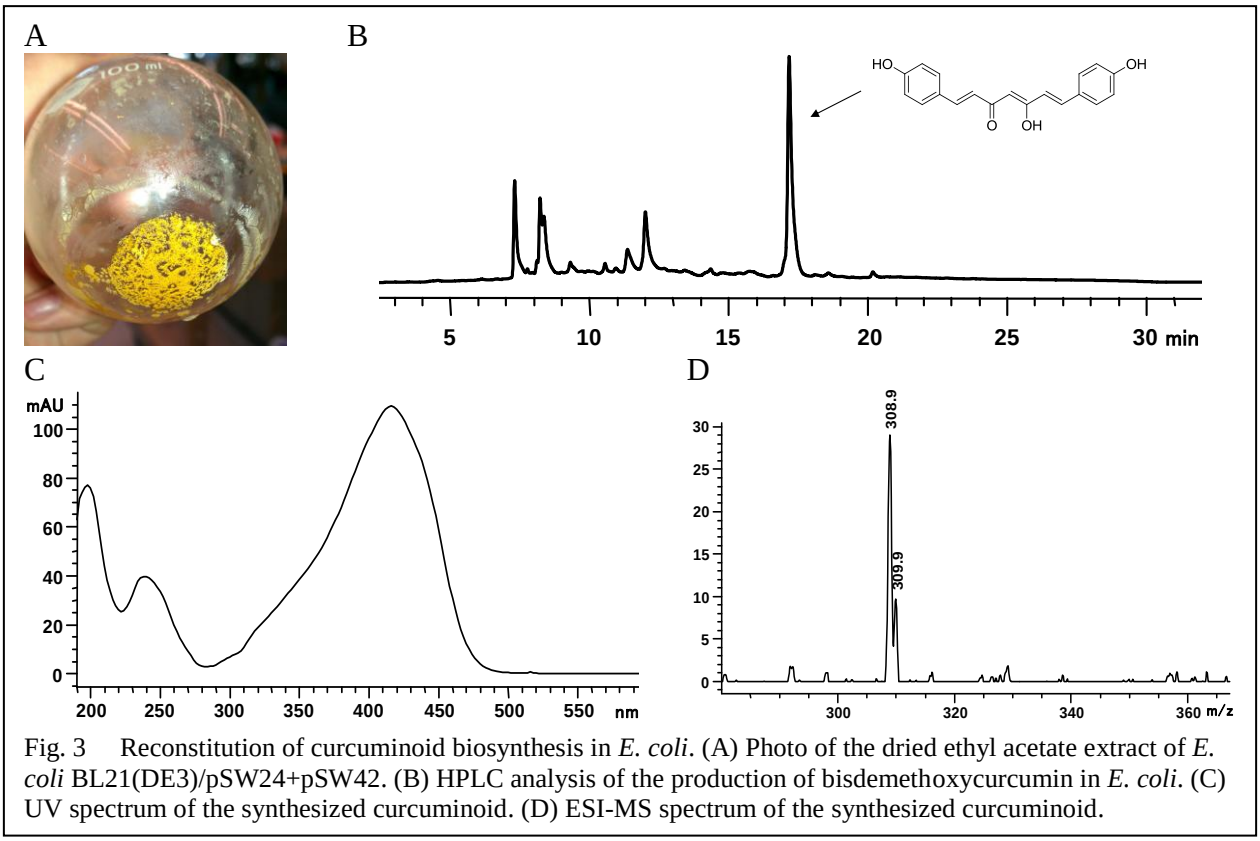


Fig. 3 Reconstitution of curcuminoid biosynthesis in *E. coli*. (A) Photo of the dried ethyl acetate extract of *E. coli* BL21(DE3)/pSW24+pSW42. (B) HPLC analysis of the production of bisdemethoxycurcumin in *E. coli*. (C) UV spectrum of the synthesized curcuminoid. (D) ESI-MS spectrum of the synthesized curcuminoid.

of this compound is consistent with those of curcuminoids (Fig. 3C). ESI-MS revealed that the molecular weight of this compound is 308 (Fig. 3D), confirming that it is bisdemethoxycurcumin. Therefore, we were able to obtain two biosynthetic enzymes from plants and reconstitute curcuminoid biosynthesis in *E. coli*. This provides a great starting point to engineer the biosynthesis of curcuminoids in LAB.

Table 1 Plasmids constructed in this work

Name	Description	Restriction Sites
pSW1	*stts*-RBS in pJET1.2	PstI/XbaI
pSW2	*stts*-RBS in pJET1.2	AgeI/XbaI
pSW3	*stts*-RBS in pTRKH2-769	PstI/XbaI
pSW5	*gfp*-RBS in pMSP3535H3	AgeI/XbaI
pSW6	*stts*-RBS in pMSP3535H3	AgeI/XbaI
pSW7	*gfp*-RBS in pJET1.2	PstI/XbaI
pSW8	*Sc-indC*-RBS in pJET1.2	NruI/XbaI
pSW10	*gfp*-RBS in pTRKH2-769	PstI/XbaI
pSW11	*sfp*-RBS in pJET1.2	NruI/XbaI
pSW12	*Sc-indC*-RBS in pMSP3535H3	NruI/XbaI
pSW16	*sfp-indC*-RBS in pMSP3535H3	NruI/XbaI
pSW19	*gfp* in pJET1.2	SmaI/XbaI
pSW20	*stts* in pJET1.2	SmaI/XbaI
pSW21	*stts* in pMG36e	SmaI/XbaI
pSW22	*gfp* in pMG36e	SmaI/XbaI
pSW23	*Sc-indC*-RBS in pJET1.2	XbaI/HindIII
pSW24	*cus* in pET28a	BamHI/NdeI
pSW26	*sfp* in pJET1.2	NruI/XbaI
pSW27	*Sc-indC* in pJET1.2	AvrII/XbaI
pSW28	*Sc-indC* in pMG36e	XbaI/HindIII
pSW29	*Sc-indC* in pMSP3535H3	AvrII/XbaI
pSW30	*sfp-indC* in pMG36e	XbaI/XbaI
pSW31	*Sc-indC*-RBS in pJET1.2	AvrII/XbaI
pSW33	*cus* in pJET1.2	XbaI/HindIII
pSW35	*cus*-RBS in pJET1.2	PstI/HindIII
pSW36	*4cl1* in pJET1.2	SmaI/XbaI
pSW39	*gfp* in pJET1.2	AvrII/XbaI
pSW40	*cus* in pJET1.2 (with N-terminal His_6-tag)	XbaI/HindIII
pSW42	*4cl1* in pACYCDEUT-1	NdeI/XhoI
pSW43	*cus* in pMG36e	XbaI/HindIII
pSW46	*cus* in pMG36e (with N-terminal His_6-tag)	XbaI/HindIII
pSW48	*pal1* in pJET1.2	PstI/NotI
pSW49	*pal3* in pJET1.2	PstI/NotI
pSW50	*pal4* in pJET1.2	PstI/NotI
pSW52	*4cl1* in pJET1.2	NdeI/XhoI
pSW54	*4cl1* in pACYCDuet-1	NdeI/XhoI
pSW56	*pal1* and *4cl1* in pACYCDuet-1	PstI/NotI; NdeI/XhoI
pSW57	*pal3* and *4cl1* in pACYCDuet-1	PstI/NotI; NdeI/XhoI

pSW58	*pal4* and *4cl1* in pACYCDuet-1	PstI/NotI; NdeI/XhoI
pZJ167	*csyA* in pJET1.2	NruI/AgeI
pZJ169	*csyA* in pMSP3535H3	NruI/AgeI
pZJ171	*csyB* in pJET1.2	AgeI
pZJ175	*csyB* in pMSP3535H3	AgeI
pZJ184	*csyA*-RBS in pJET1.2	XbaI/PstI
pZJ185	*csyB*-RBS in pJET1.2	SmaI/PstI
pZJ187	*csyA*-RBS in pJET1.2	XbaI/AgeI
pZJ188	*csyB*-RBS in pJET1.2	AgeI
pZJ189	*csyA*-RBS in pMG36e	XbaI/PstI
pZJ190	*csyB*-RBS in pMSP3535H3	AgeI
pZJ193	*csyB*-RBS in pMG36e	SmaI/PstI

2. Development of a novel *in vivo* reporter assay for efficient PAL

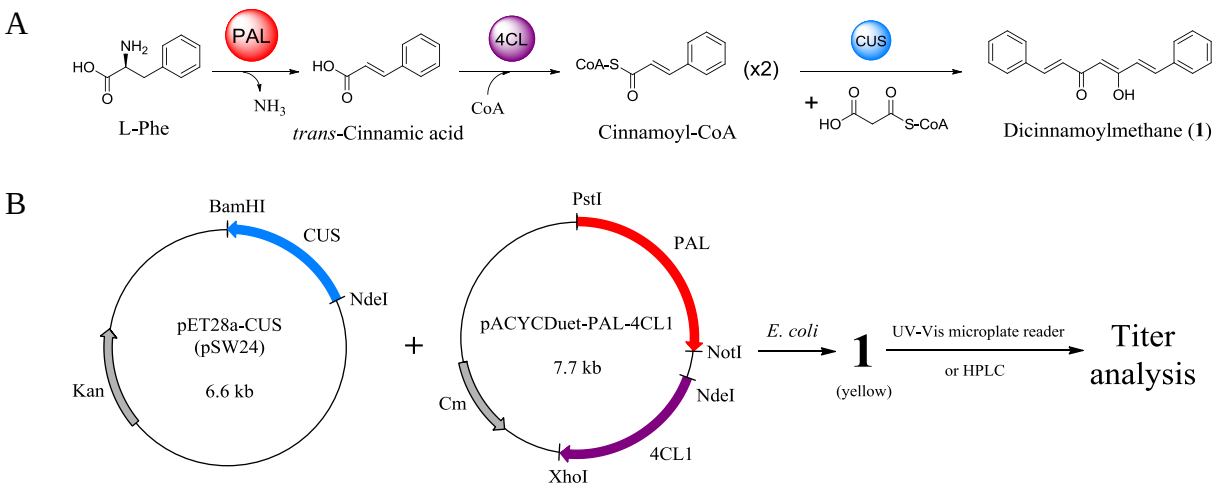


Fig. 4 Design of a reporter assay for PALs. (A) Biosynthetic pathway of **1** from L-Phe by the sequential actions of PAL, 4CL and CUS. (B) A reporter assay for screening of PALs in *E. coli*. The system consists of two plasmids. The first one contains CUS and the second one has 4CL1. A candidate PAL gene will be ligated into the second vector between PstI and NotI. Co-expression of the two plasmids will lead to the biosynthesis of **1**, which is visible for its yellow color and can be analyzed by HPLC or UV-Vis microplate reader.

PAL is an important enzyme that links primary metabolism to secondary metabolism. Its efficiency is often a critical factor that affects the overall flux of a related metabolic pathway and

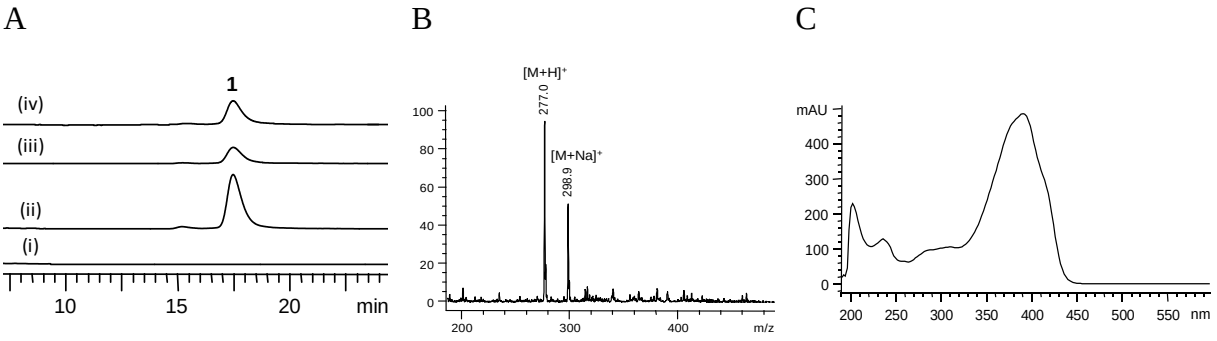


Fig. 5 HPLC-MS analysis of the production of **1** in engineered *E. coli*. (A) HPLC traces of the methanol extract of the cell pellets of *E. coli*/pSW24+pSW54 (i), *E. coli*/pSW24+pSW56 (ii), *E. coli*/pSW24+pSW57 (iii) and *E. coli*/pSW24+pSW58 (iv). (B) ESI-MS (+) spectrum of **1**. (C) UV absorption spectrum of **1**.

the titer of the final products such as curcuminoids. In Section 1, we were able to reconstitute the biosynthesis of bisdemethoxycurcumin in *E. coli* with the supplement of courmaric acid. Addition of PAL into this pathway will allow direct biosynthesis of curcuminoids from amino acids. To this end, we established a novel and efficient visible reporter assay for screening efficient PALs in *E. coli* based on the curcuminoid biosynthetic pathway. The candidate PALs were co-expressed with 4CL1 and CUS in *E. coli* BL21(DE3) to form a dicinnamoylmethane (**1**) biosynthetic pathway (Fig. 4A). Taking advantage of the yellow color of the product, a microplate-based assay was designed to measure the titer of **1**, which was validated by HPLC analysis (Fig. 4B). The different titers of the product reflect the overall performance (expression and enzymatic efficiency) of the individual PALs in *E. coli*.

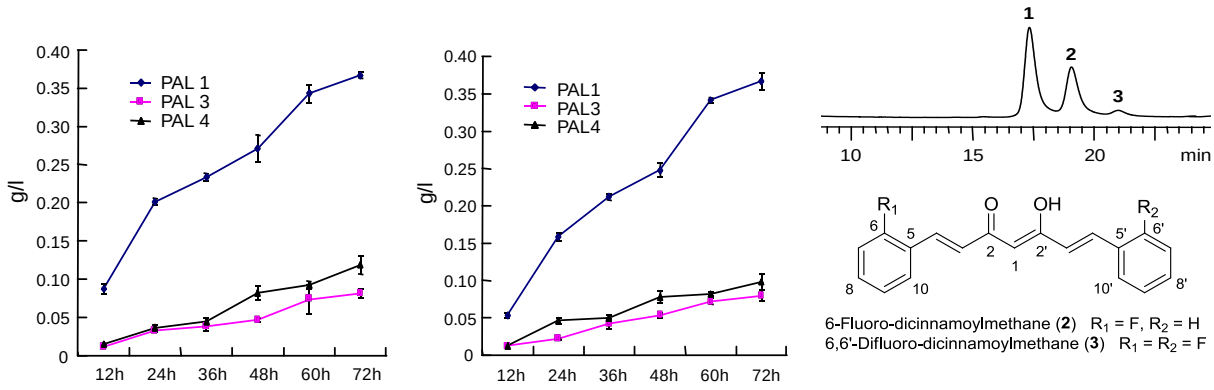


Fig. 6 Time course analysis of the production of **1** and precursor-directed biosynthesis of fluorinated dicinnamoylmethane derivatives. (A) Time course analysis by a UV-Vis microplate reader at 390 nm. (B) Validation of the microplate-based assay by HPLC analysis at 390 nm. (C) HPLC analysis of the production of fluorinated dicinnamoylmethane derivatives **2** and **3** in *E. coli*.

Using this system, we have screened three PALs (PAL1, PAL3 and PAL4) from *Trifolium pretense*. Specifically, CUS was ligated into pET28a to yield pSW24 as described above. 4CL1 and a candidate PAL gene were ligated into pACYCDuet-1 to yield pSW56, pSW57 and pSW58. The resulting plasmids were co-expressed with pSW56, pSW57 and pSW58, respectively, in *E. coli*. LC-MS analysis revealed that all three PALs were functionally expressed in *E. coli* to yield the production of **1** (Fig. 5). Among these three plant PALs, PAL1 showed the best performance in *E. coli* according to the microplate-based assay (Fig. 6A) and HPLC analysis (Fig. 6B). The engineered *E. coli* strain containing PAL1, 4CL1 and CUS led to the production of **1** at a high level of 0.36 g/l. We also used qRT-PCR to compare the expression level of the three plant PALs in *E. coli*/pSW24+pSW56, *E. coli*/pSW24+pSW57 and *E. coli*/pSW24+pSW58, respectively. As shown in Table 2, the qRT-PCR results indicated that the expression level of PAL1 was higher than the other two, and the expression level of the PAL3 was the lowest among these three PALs. This is consistent with the analysis results of the titers of **1** in the corresponding *E. coli* strains, although the latter is the combined effects of both the expression level and catalytic efficiency of PALs.

Table 2 Relative expression level of three different plant PALs in *E. coli*

Enzyme	ΔCt
PAL1	-4.06±0.46
PAL3	-1.10±0.34
PAL4	-3.50±0.36

Supplement of 2-fluoro-L-phenylalanine into the fermentation broth of *E. coli*/pSW24+pSW56 yielded two fluorinated dicinnamoylmethane derivatives (Fig. 6C). The products were characterized on the basis of NMR and ESI-MS data as 6,6'-difluoro-dicinnamoylmethane (**2**) and 6-fluoro-dicinnamoylmethane (**3**), respectively, of which the latter is a new curcuminoid. This work not only provides a very useful assay for screening PALs, but also yielded an efficient PAL for engineered biosynthesis of curcuminoids in LAB.

3. Expression of GFP in *Lactobacillus casei* NRRL B-441

LAB have been used for thousands of years to make various foods. With the rapid development of genetic engineering techniques, LAB can be engineered for increased industrial functionality.[18] In order to reconstitute curcuminoid biosynthesis in LAB, we will need to establish an effective expression platform.

To identify a useful expression vector in LAB, we chose the *gfp* gene from the jellyfish *Aequoria victoria* as a reporter. We tested three known *E. coli*/LAB shuttle vectors, including pMSP3535H3, pTRKH2-769 and pMG36e. We cloned the *gfp* gene (with or without RBS in the primers) into these vectors, yielding pSW5, pSW10 and pSW22 (Table 1), respectively. The resulting plasmids were transformed into *L. casei* NRRL B-441 through electroporation. The transformants were grown in deMan, Rogosa and Sharpe (MRS) broth supplemented with 5 μg/ml erythromycin at 37 °C for 2 days. The cells were then harvested by centrifugation and washed by water twice for analysis of GFP expression. We found that pSW32 (pMG36e-*gfp*), in which the *gfp* gene is under the constitutive P32 promoter (Fig. 7A), showed the strongest fluorescence level. The cells of NRRL B-441/pSW22 and NRRL B-441 blank control were diluted to the same OD_{600} level, and then measured on a Biotek Synergy 4 (Biotek, Winooski, VT) plate reader (Fig. 7B). *E. coli* XL1-Blue/pSW19 was used as a positive control. Our results showed that pMG36e is an effective vector for protein expression in *L. casei*.

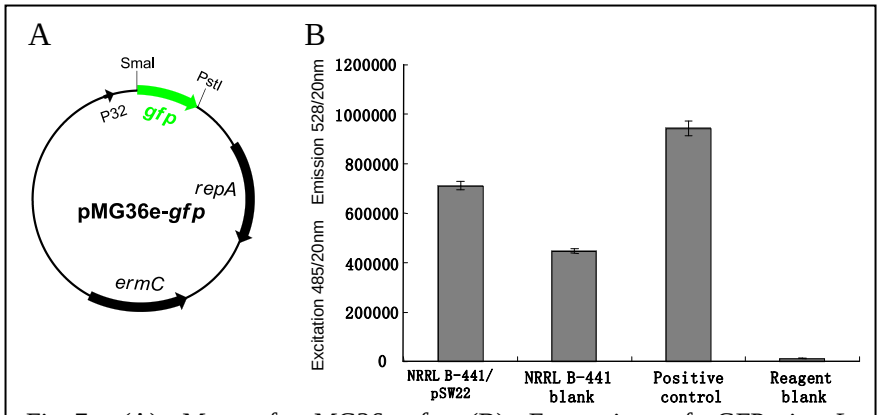


Fig. 7 (A) Map of pMG36e-*gfp*. (B) Expression of GFP in *L. casei*/pMG36e-*gfp*.

4. Construction of expression plasmids for the biosynthesis of curcuminoids and other natural products in LAB

Because engineered biosynthesis of natural products in LAB has not been well investigated, we intended to test several simpler biosynthetic pathways, while working toward to constructing a three-enzyme pathway in LAB. Natural product biosynthetic pathways have been extensively studied in the last two decades. Among the identified natural product biosynthetic enzymes, polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) are two of the most commonly observed and well investigated. My group has been focusing on PKSs and NRPSs, and we have characterized a number of enzymes that may be reconstituted in LAB. In this work, several PKSs and one NRPS gene has been cloned into *E. coli*/LAB shuttle vectors for functional reconstitution in LAB.

4.1 Biosynthesis of flaviolin

Our group has recently characterized a flaviolin biosynthetic pathway from *Streptomyces*

toxytricini NRRL 15443 that consists of a type III PKS (StTS), a monooxygenase (StMO), and two cytochrome P450 enzymes (StP450-1 and StP450-2).[19] StTS is homologous to RppA, a 1,3,6,8-tetrahydroxynaphthalene (THN) synthase from *Streptomyces griseus*.[20] Through both in *vitro* and *in vivo* studies, we have confirmed that StTS takes five units of malonyl-CoA to synthesize THN, which then can be oxidized by StMO or O_2 to form the red pigment, flaviolin (Figs. 8A and 8B). StP450-1 and StP450-2 can oxidize flaviolin to form oligomers. We have reconstituted flaviolin biosynthesis in *E. coli* through heterologous expression of StTS (Figs. 8B and 8C).[19] Encouraged by this, we have also further cloned this gene into pTRKH2-769, pMSP3535H3 and pMG36e to yield pSW3, pSW6 and pSW21 (Table 1), respectively, for reconstitution of the biosynthesis of this red compound in LAB.

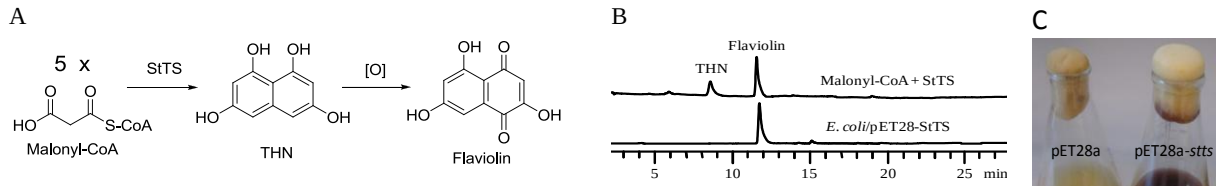


Fig. 8 (A) Biosynthetic pathway of flaviolin by StTS. (B) HPLC analysis of *in vitro* (top) and *in vivo* (bottom) biosynthesis of flaviolin by StTS in *E. coli*. (C) Picture of flaviolin production in *E. coli* BL21(DE3).

4.2 Biosynthesis of indigoidine

Indigoidine is a blue pigment in bacteria. We have identified a gene cluster from *Streptomyces chromofuscus* ATCC 49982, which contains three genes: *Sc-indA*, *Sc-indB* and *Sc-indC*.[21] The *Sc-indC* gene encodes a NRPS that is believed to synthesize indigoidine. We have cloned the *Sc-indC* gene into pET28a (Fig. 9A) and overexpressed the protein in *E. coli* BAP1. The blue pigment can be easily observed in the colonies and fermentation broth (Fig. 9B). LC-MS analysis confirmed the production of indigoidine (Fig. 9C, MS data not shown). We have also cloned *Sc-indC* into pRM5, an *E. coli/Streptomyces* shuttle vector, and reconstituted indigoidine biosynthesis in *Streptomyces lividans* K4 (data not shown). Thus, we have reconstituted this NRPS in two different bacterial hosts. The biosynthetic pathway of indigoidine from L-glutamine is proposed in Fig. 9D. The bright blue color of this compound makes it a useful reporter for expression testing in LAB. Accordingly, we have cloned *Sc-indC* into different *E. coli*/LAB shuttle vectors to yield a series of expression plasmids, including pSW12, pSW28 and pSW29 (Table 1). To effectively activate the NRPS, we have also added an additional gene, *sfp* from a

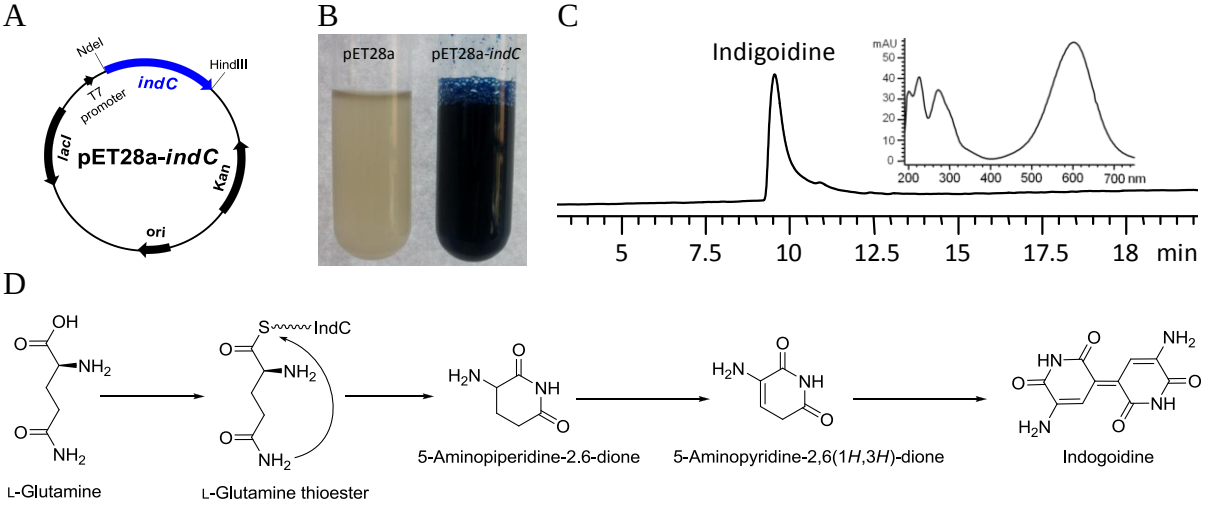


Fig. 9 (A) Map of pET28a-*indC*. (B) Picture of the production of indigoidine in *E. coli* BAP1. (C) HPLC analysis of the fermentation broth of *E. coli* BAP1/pET28a-*indC* at 600 nm and the UV spectrum of indigoidine. (D) Proposed biosynthetic pathway of indigoidine.

Bacillus subtilis that encodes a phosphopantetheinyl transferase, to yield two new plasmids pSW16 and pSW30 (Table 1). All these plasmids will be transformed into *L. casei* NRRL B-441 for product analysis.

4.3 Construction of additional expression plasmids

The construction of plasmids for StTS and Sc-IndC will allow us to easily analyze the production of the corresponding color compounds. To express CUS in LAB, we have cloned *cus* (with or without an N-terminal His_6-tag) into pMG36e to yield pSW43 and pSW46. The designed His_6-tag is for western blot analysis of expression of CUS in LAB. When the expression of CUS is confirmed, *4cl* and a phenylalanine ammonia-lyase (PAL) gene will be introduced into pSW43 to reconstitute the biosynthesis of curcuminoids.

In the meantime, because CUS is a eukaryotic type III PKS, we also constructed several additional plasmids to test the expression of other eukaryotic type III PKSs in LAB. We have recently characterized two new fungal type III PKSs, CsyA [22] and CsyB, from *Aspergillus oryzae*, which synthesize different fungal pyrone metabolites. We have cloned the corresponding genes into pMG36e and pMSP3535H3, yielding pZJ189, pZJ169, pZJ193 and pZJ190 (Table 1), respectively. These plasmids will provide more examples for reconstitution of eukaryotic PKSs in LAB.

5. Analysis of plasmids in LAB by colony PCR

Transformation of foreign plasmids into LAB such as *L. casei* NRRL B-441 may give some fake colonies. To identify the correct transformants, we developed a colony PCR approach. Using this technique, we can easily identify the desired transformants. An example of confirming the successful transformation of pMG36e-*csyB* (pZJ193) is given. Colonies were picked and grown in liquid MRS medium supplemented with 5 μg/ml erythromycin. *L. casei* NRRL B-441 was grown in the same medium without any antibiotics as control. After 2 days of incubation, the broths were used as the templates for PCR. As shown in Fig. 10, it is obvious that a single PCR product of *csyB* (1,194 bp) was observed in the transformant, but not in the blank strain. This modified colony PCR technique will allow us to easily and accurately determine a correct LAB transformant.

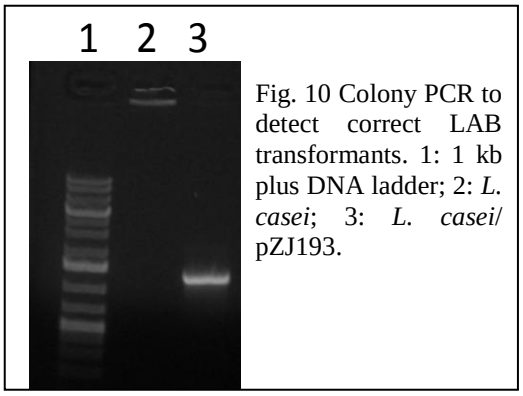


Fig. 10 Colony PCR to detect correct LAB transformants. 1: 1 kb plus DNA ladder; 2: *L. casei*; 3: *L. casei*/ pZJ193.

Task 2: Comparative analysis of yogurt products resulting from the wide type and engineered strain

Task 2 is dependent on task 1. Since task 1 has not been completely accomplished, this task was not carried out. However, it should be straightforward after we get the curcuminoids-producing LAB.

Key Research Accomplishments

1. Curcuminoid biosynthesis has been reconstituted in a heterologous host, *E. coli* BL21(DE3).
2. A novel *in vivo* reporter assay has been developed for screening efficient phenylalanine ammonia-lyases.
3. A useful expression platform that contains the pMG36e vector and the LAB host *L. casei* NRRL B-441 has been established.
4. A series of expression plasmids containing different natural product biosynthetic enzymes has been constructed.

5. An efficient colony PCR technique has been developed to analyze correct LAB transformants.

Reportable Outcomes

Publications:

Yu, D., Xu, F., Zeng, J., Zhan, J. (**2012**): Type III polyketide synthases in natural product biosynthesis. *IUBMB Life* **64**(4), 285–295.

Wang, S., Zhang, S., Zhou, T., Zeng, J., Zhan, J. (**2013**): Design and application of an *in vivo* reporter assay for phenylalanine ammonia-lyase. *Applied Microbiology and Biotechnology* (submitted).

Presentations:

Wang, S., Zeng, J., Zhan, J. Towards engineered biosynthesis of curcuminoids in lactic acid bacteria. 2012 Institute of Biological Engineering Western Regional Meeting, October 26, 2012, Logan, UT (oral and poster presentations).

Conclusion

We have reconstituted curcuminoid biosynthesis in a heterologous host, *E. coli*. The genes we have amplified were shown to be functional and efficient. By testing different *E. coli*/LAB shuttle vectors, we chose pMG36e as a starting vector. Using this vector, we have successfully expressed GFP in *L. casei* NRRL B-441. We have also constructed a series of expression plasmids containing different biosynthetic genes for various natural products, including those for curcuminoids. Our work has demonstrated the feasibility of expressing curcuminoid biosynthetic genes in LAB and has built a platform for further engineered biosynthesis of curcuminoids for preventive treatment of food allergies.

References

(1) Aggarwal, B. B.; Sundaram, C.; Malani, N.; Ichikawa, H. *Adv. Exp. Med. Biol.* 2007, *595*, 1.
(2) Goel, A.; Kunnumakkara, A. B.; Aggarwal, B. B. *Biochem. Pharmacol*. 2008, *75*, 787.
(3) Hatcher, H.; Planalp, R.; Cho, J.; Tortia, F. M.; Torti, S. V. *Cell. Mol. Life Sci.* 2008, *65*, 1631.
(4) Debjit, B.; Chiranjib; Kumar, K. P. S.; Margret, C.; Jayakar, B. *Arch. Appl. Sci. Res.* 2009, *1*, 86.
(5) Aggarwal, B. B.; Harikumar, K. B. *Int. J. Biochem. Cell Bio.* 2009, *41*, 40.
(6) Aggarwal, B. B.; Kumar, A.; Bharti, A. C. *Anticancer Res.* 2003, *23*, 363.
(7) Hamaguchi, T.; Ono, K.; Yamada, M. *CNS Neurosci. Ther.* 2010, *16*, 285.
(8) Kurup, V. P.; Barrios, C. S. *Mol. Nutr. Food Res.* 2008, *52*, 1031.
(9) Lee, S. Y.; Yuk, D. Y.; Song, H. S.; Yoon, D. Y.; Jung, J. K.; Moon, D. C.; Lee, B. S.; Hong, J. T. *Eur. J. Pharmacol.* 2008, *582*, 17.
(10) Moon, D. O.; Kim, M. O.; Lee, H. J.; Choi, Y. H.; Park, Y. M.; Heo, M. S.; Kim, G. Y. *Biochem. Biophys. Res. Commun.* 2008, *375*, 275.
(11) Lockey, R. F., *http://clinicaltrials.gov/ct2/show/NCT01179256*.
(12) Biffi, A.; Coradini, D.; Larsen, R.; Riva, L.; Di Fronzo, G. *Nutr. Cancer* 1997, *28*, 93.
(13) Klijn, N.; Weerkamp, A. H.; De, V. W. M. *Appl. Environ. Microbiol.* 1995, *61*, 2771.
(14) Bezkorovainy, A. *Am. J. Clin. Nutr.* 2001, *73*, 399S.
(15) Kawal, Y.; Suegara, N.; Shimohashi, H. *Microbiol. Immunol.* 1982, *26*, 363.
(16) Morita, H.; Wanibuchi, K.; Nii, H.; Kato, R.; Sugio, S.; Abe, I. *Proc. Natl. Acad. Sci. U. S. A.* 2010, *107*, 19778.

(17) Katsuyama, Y.; Matsuzawa, M.; Funa, N.; Horinouchi, S. *J. Biol. Chem.* 2007, *282*, 37702.
(18) Bron, P. A.; Kleerebezem, M. *Bioeng. Bugs* 2011, *2*, 80.
(19) Zeng, J.; Decker, R.; Zhan, J. *Appl. Biochem. Biotechnol.* 2012, *166*, 1020.
(20) Funa, N.; Ohnishi, Y.; Fujii, I.; Shibuya, M.; Ebizuka, Y.; Horinouchi, S. *Nature* 1999, *400*, 897.
(21) Yu, D.; Xu, F.; Valiente, J.; Wang, S.; Zhan, J. *J. Ind. Microbiol. Biotechnol.* 2012, *40*, 159.
(22) Yu, D.; Zeng, J.; Chen, D.; Zhan, J. *Enzyme Microb. Tech*. 2010, *46*, 575.